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ABSTRACT

Rev-erbα, a component of the circadian clock, has also been known as a nuclear receptor that lacks activation function domain 2, functioning as a ligand-dependent transcriptional repressor. However, we recently reported that Rev-erbα activates *connexin43* transcription by forming a complex with Sp1. Here we show that heme, a REV-ERB ligand, is dispensable for this novel mechanism and that Rev-erbβ, having homologies with Rev-erbα, does not activate *connexin43*, but competes with the Rev-erbα/Sp1. The A/B region of Rev-erbα, which is not conserved in Rev-erbβ, is a crucial activating domain, while the ligand binding domain, conserved in Rev-erbβ, functions as a competitor.

Key words: Connexin43/Nuclear receptor/Rev-erbα/ Rev-erbβ /Sp1

Abbreviations: connexin43, Cx43; A/B region, AB, DNA binding domain, DBD; hinge region, H; ligand binding domain, LBD; Estrogen Receptor, ER

INTRODUCTION

Rev-erbα and Rev-erbβ, initially identified as orphan nuclear receptors Nr1d1 and Nr1d2, are also components of the circadian clock, have important roles in metabolism and cellular differentiation [1-4]. The circadian clock is genetic machinery that generates autonomous genetic oscillations in systemic organs by coordination of transcriptional activators (Bmal1 and Clock) and repressors (Per1–3 and Cry1, 2) that regulate other genes by binding to E boxes in promoter regions [5,6]. Concurrently, Rev-erbα/β and Rorα/β/γ has been shown to tune the amplitude and the phase of the circadian clock, or control transcription of other non-clock genes, by binding to RORE sites of target genes [1,3,7,8].

Nuclear receptors modulate transcriptional activity by binding to specific ligands such as steroid hormones, thyroid hormone, vitamin D and retinoids, and function either as activators or repressors by recruiting co-activators or co-repressors, respectively [9,10]. Rev-erbα and Rev-erbβ have been considered to be ligand-dependent transcriptional repressors since they lack a canonical activation function domain 2 (AF-2) [11,12] and their repressive effects by recruiting nuclear receptor co-repressor/histone deacetylase 3 depend on their ligand of heme [13-16].

However, in a sharp contrast to these previous notions, we have revealed that Rev-erbα functions as an activator for *connexin43* gene (*Cx43*) by forming a complex with Sp1 in HEK293T cells and bladder smooth muscle cells [17]. Importantly, this effect required proximal Sp1 sites, but no RORE sequences, on the *Cx43* promoter.

Sp1 is an Sp/KLF family transcriptional factor that regulates expression of multiple genes with GC-rich sequences, i.e., Sp1 binding sites, on their promoter regions [18,19]. The Sp1-dependent transactivation of GC-rich promoter sequences is reported with various nuclear proteins as well as basal transcription factors [11]. Interaction between Sp1 and nuclear receptors are reported with RAR, RXR, ERs and PPAR, which possess common structures of nuclear receptor domains including an A/B region (AB), a DNA binding domain (DBD), a hinge region (H) and a ligand binding domain (LBD) [20-22].

Here, we investigate the detailed mechanism by which Rev-erbα and Sp1 complex to transactivate the *Cx43* promoter in comparison with Rev-erbβ, and clarify the roles of Rev-erbα domains for the process.

METHODS

Plasmid constructs. The mouse pGL-2-Cx43 promoter-reporter (pCx43 -1686/+165-luc), constructed by Dr. S.J. Lye [23], was a kind gift from Dr. J. Yao (University of Yamanashi, Yamanashi, Japan). The pRL-TK (Promega, Madison, WI) were used as a transfection efficacy control. The Sp1 expression vector, constructed by Dr. Suske [24] was a kind gift from Dr. Toguchida (Frontier Medical Sciences, Kyoto University, Kyoto, Japan). The expression vector of Rev-erbα and Rev-erbβ was purchased from Open Biosystems (Huntsville, AL). Site-directed mutagenesis, deletion and addition of aimed sequences were performed using a mutagenesis basal kit (Takara) according to the manufacturer's protocol. These mutants were all verified by sequencing.

Promoter-reporter assay. Reporter plasmids with various expression vectors were transfected into HEK293T cells in 24-well plates using Fugene6 (Roche) in DMEM with 10% fetal calf serum (FCS) according to the manufacturer's protocol. pCx43-luc or pGL2-basic 100 ng and pTK-RL 5 ng were transfected with various amounts of expression vectors (total 250 ng). Plasmid dosage was kept constant by EGFP-N1 vector. Lysates were harvested 48 hours post-transfection, and the luciferase activity was measured using a dual luciferase assay reagent (Promega). For the hemin treatment, medium was changed to various concentration of hemin in DMEM with 10% FCS at 24 hour after the transfection and kept for 24 hour. For SR8278 (Sigma) and GSK4112 (Sigma) treatment, 24 hours after the transfection medium was changed to DMEM with 10% FCS containing 10μM of each reagent and incubated for additional 24 hours.

Immunoblotting. Preparation of whole-cell lysates from HEK293T cells, and the immunoblotting procedure were performed as previously described [17,25]. Briefly, cells were lysed in radio-immunoprecipitation assay (RIPA) buffer containing protease inhibitors. The protein content of the cell lysates was measured using the BioRad Protein Assay Kit. Cell lysates were resolved by sodium dodecyl sulfate polyacrylamide electrophoresis and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membranes were incubated with antibodies for Sp1 (Millipore, Milford MA, 1:2000), HA (Abcam, 1:8000), DDDDK (MBL, Aichi, Japan 1:2000), Rev-erbα (Cell Signaling Technology [CST], Beverly, MA, 1:500), Cx43 (Sigma, St Louis, MO, 1:1000), Bmal1 (Santa Cruz Biotechnology, Santa Cruz, CA 1:200) and GAPDH (CST, 1:2000). The Rev-erbα antibody of CST (2124) was for 20 amino acids between 200 and 300 from the N terminus, including the H region. After incubation with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (Pierce, Rockford, IL), immunoreactive proteins were visualized using a Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

Co-immunoprecipitation assay. Preparation of nuclear extracts and

co-immunoprecipitation were performed as previously described [17]. Briefly nuclear extracts were prepared from HEK293T cells in 10 cm dishes, transfected with HA-Rev-erbα or HA-Rev AB (3.75 μg), and DDDDK-Sp1 (3.75 μg) expression vectors for 48 hours, using Nuclear Complex Co-IP kit (Active Motif, Carlsbad, CA). The 100 μg of nuclear extracts were incubated with 4 μg of antibodies for HA and control rabbit IgG (Zymed) in 500 μl of low IP buffer overnight at 4 °C with rotation followed by addition of 30 μl of Dynabeads Sheep anti-Rabbit IgG (Veritas, Tokyo, Japan) for 1 hour. After washing with low IP buffer, the binding protein was eluted in 40 μl of RIPA buffer for immunoblotting. The 2 μg of nuclear extracts were used as input.

Statistical analysis. We used one-way ANOVA followed by Tukey's *post-hoc* test for the multiple comparisons or by Dunnett's *post-hoc* test for comparing to the control in the promoter-reporter assay, calculated with SPSS ver.11.0.1J software (SPSS Inc., Chicago, IL). A P value < 0.05 was accepted as significant.

RESULTS AND DISCUSSION

Ligand is dispensable for activation by Rev-erbα with Sp1

Firstly, to investigate whether the transactivation of *Cx43* promoter by

Rev-erbα/Sp1 complex is controlled by heme, the ligand of Rev-erbα as a nuclear receptor, we applied various concentrations of heme to the HEK293T cells transfected with Rev-erbα/Sp1. Exogenously applied heme showed little impact on the *Cx43* promoter activation (**Fig. 1A**), while it enhanced the suppressive effect on the abundance of Bmal1 protein as reported (**Fig. 1B)** [13,15]. In addition, SR8278, an antagonist of REV-ERB, was applied to block the action of endogenous agonist heme, which is always present in the cells [26]. It also had little effect on the *Cx43* promoter activation, which was not influenced by addition of GSK4112, an agonist of REV-ERB (**Supplementary Fig. S1**). Notably, although the ligand binding domain (LBD) of Rev-erbα has been reported to be crucial for recruiting co-repressors [14,15], a deletion mutant of LBD of Rev-erbα (Rev ΔLBD) still retained transactivation activity for the *Cx43* promoter (**Fig. 1C**). In contrast to the suppressive effect of Rev-erbα, the ligand is not considered to play a significant role in the transactivation mechanism.

Rev-erbβ competes with Rev-erbα and Sp1 complex

Next, to investigate whether Rev-erbβ transactivates the *Cx43* promoter in a similar manner as Rev-erbα with Sp1, we drove expression of Rev-erbβ in conjunction with a *Cx43* promoter-reporter. However, Rev-erbβ scarcely affected the promoter

activity of *Cx43* even when co-expressed with Sp1 (**Fig. 2A**). Intriguingly, Rev-erbβ competed with Rev-erbα in the presence of Sp1 for the transactivation of the *Cx43* promoter (**Fig. 2B**). This result is completely different from the previous notion that Rev-erbα and Rev-erbβ have nearly identical functions in the liver [27]. These results prompted us to investigate the precise mechanism of *Cx43* promoter transactivation by Rev-erbα with Sp1.

The A/B region of Rev-erbα is an activation domain.

Alignment comparison of Rev-erbα with Rev-erbβ disclosed that they have a different A/B region but a similar DNA binding domain (DBD), a hinge region (H) and a C terminal region of their LBD (**Supplementary Fig. S2**). According to their similarities and differences, we generated various domain oriented deletion mutants of Rev-erbα to examine the domains responsible for the activation of *Cx43* (**Fig. 3A**). The promoter-reporter assay revealed that, a part of the N terminal region of Rev-erbα (8-49) and the LBD were not crucial for the activation of the *Cx43* promoter, while the A/B region, DBD and H region were indispensable (**Fig. 3B**). These effects were also confirmed at the protein level by immunoblotting (**Fig. 3C**). In accordance with previous reports [1,14], wild-type (WT) Rev-erbα successfully suppressed Bmal1

protein expression. Notably, in contrast to the transactivation of the *Cx43* promoter, co-transfection of a deletion mutant of the A/B region (ΔAB) still had a suppressive effect on Bmal1 expression, while those with deletions of the DBD, H region or LBD (ΔDBD, ΔH and ΔLBD) failed to suppress it.

In light of these results, we focused on the A/B region as an activation domain and generated a DNA construct for expression of this region alone (HA-Rev AB). Indeed, the HA-Rev AB still transactivated the *Cx43* promoter transfected with Sp1 (**Fig. 3D**) and the HA-Rev AB was co-immunoprecipitated with Sp1 (**Fig. 3E**).

The LBD of Rev-erbα is a competition domain.

Rev ΔAB, a mutant Rev-erbα without A/B region, not only failed to activate, but inhibited the transactivation of *Cx43* promoter by Rev-erbα with Sp1 in a dose dependent manner (**Fig. 4A**), indicating that Rev ΔAB contains a competition domain for Rev-erbα/Sp1 complex to transactivate the *Cx43* promoter.

To identify the competition domain of Rev-erbα, we assessed the effect of Rev ΔH, Rev DBDLBD (a mutant of Rev-erbα only with the DBD and LBD) or Rev DBD (a mutant of Rev-erbα only with the DBD) with Rev-erbα/Sp1 (**Supplementary Fig. S3**). The competition was observed in Rev ΔH and Rev DBDLBD but not in Rev DBD,

indicating that not the H region or DBD but the LBD could be the required domain. Indeed, Rev LBD (a mutant of Rev-erbα only with LBD) competed with the Rev-erbα/Sp1 (**Fig. 4B**). Therefore, the LBD is involved in the transactivation of the *Cx43* promoter by Rev-erbα/Sp1 complex as a potential competition domain.

Thus, different amino acid arrangements in the A/B activation domain regions of Rev-erbα and Rev-erbβ may underlie their differential activation of the *Cx43* promoter. Conversely, high compatibility in LBD between these two Rev-erb subtypes may underlie the competitive effect of by Rev-erbα by Rev-erbβ (**Fig. 4C**). We should note that this relationship between Rev-erbα and Rev-erbβ is similar to the relationship between Estrogen Receptor (ER) α and ERβ. The importance of the A/B region of nuclear receptors for associating with Sp1 has also been postulated for this ER family. Saville and coworkers reported that the ERα possesses a transactivation function, but ERβ does not, while a chimeric ERα/β, possessing the A/B region of ERα and other domains of ERβ, also has a transactivation function on a 17-beta Estradiol 2-responsive construct containing a GC-rich promoter [28]. ERα and ERβ are also competitive on the Sp1 site of the promoter [28]. Li and coworkers demonstrated that amino acids 67-139 of ERα, a part of the A/B region, were sufficient for the transactivation by ERα/Sp1 [29].

The physiological implication of this novel competitive role for Rev-erbβ is unclear. Rev-erbβ is also a component of the circadian clock [27,30] and oscillates in the urinary bladder (**Supplementary Fig. S4**), thus one could speculate that Rev-erbβ tunes the circadian expression of *Cx43* by competing with Rev-erbα. Because the peak expression of *Rev-erbβ* is 3.6 h later than *Rev-erbα*, followed by *Cx43* (**Supplementary Fig. S4**) in the urinary bladder, Rev-erbβ may contribute to decrease the transactivation of *Cx43* after the peak point. Since the present study relies to a large extent on overexpression and reporter assays, physiological relevance and functional significance of the findings still remains to be determined.

As other limitation of the present study, we showed the specific role of Rev-erbα domains for transactivation of the *Cx43* promoter, but not the specific amino acid sequence required for the activation by Rev-erbα/Sp1 complex. The molecular structure of the complex, including the detailed interaction with Sp1 and other co-activators and/or co-repressors, which may also induce differences in effect of Rev-erbα/β [31-34], remains to be investigated to elucidate the entire mechanism of this novel transcription complex.

In summary, the present study shows that heme as the ligand is dispensable for transactivation of the *Cx43* promoter by Rev-erbα with Sp1, and that the A/B region of

Rev-erbα functions as an activation domain, while the LBD of Rev-erbα is a competition domain. This mechanism may underlie the competitive effect by Rev-erbβ on the transactivation. These findings provide a new insight into interaction between the clock regulator/nuclear receptors and Sp1.

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AUTHOR CONTRIBUTIONS

H.N. and A.K designed the experiments and composed the manuscript. H.N. performed the experiments and analyzed data. T.O and M.I helped with the experiments. Y.T. and O.O. supervised the study.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

LEGENDS

Figure 1 Ligand independent activation by the Rev-erbα and Sp1 complex. (**A**) Addition of hemin, a ligand of Rev-erbα, did not increase the effect of Rev-erbα and Sp1 on *Cx43* transcription. N=3 for each group. (**B**) Bmal1expression was decreased by addition of hemin on Rev-erbα and Sp1. (**C**) A mutant with deletion of the ligand binding domain (Δ287-610: Rev ΔLBD) still transactivated the *Cx43* promoter. **P* < 0.0001 compared with the control without transfection of Rev-erbα by one-way ANOVA followed by Dunnett's *post-hoc* test. +, 125 ng in **A** and **C**. Error bars represent s.d. in **A** and **C**. For relative levels, Rev-erbα + Sp1 + hemin 0 μM was set to 1 in **A**, and the control as Rev-erbα - Sp1 - was set to 1 in **C**.

Figure 2 Rev-erbβ competes with the Rev-erbα and Sp1 complex. (**A**) Rev-erbβ did not transactivate *Cx43* promoter. **P* < 0.0001 vs. Rev-erbβ -, Rev-erbα - Sp1 - ; †*P* < 0.0001 vs. Rev-erbβ - Rev-erbα - Sp1 + by one-way ANOVA followed by Tukey's *post-hoc* test. N=3 for each group. +, 125 ng; ++ 250 ng. (**B**) Rev-erbβ

competes with Rev-erbα and Sp1 complex for transactivation of *Cx43* promoter. N=3. *p<0.0005 vs. Rev-erbβ - Rev-erbα - Sp1 - or +; †p<0.0001 vs. Rev-erbβ - Rev-erbα + Sp1 + by one-way ANOVA followed by Tukey's *post-hoc* test. +, 50 ng; +++, 150 ng. Error bars represent s.d. in **A** and **B**. The controls without Rev-erbα and Sp1 were set as 1 in **A** and **B**.

Figure 3 A/B region dependent activation of the Rev-erbα and Sp1 complex. (**A**) Schematic representations of deletion mutants of Rev-erbα. (**B**) The AB region (8-126), DNA binding domain (DBD, 127-203) and hinge regions (H, 204-286) are crucial to activate the *Cx43* promoter. +, 125 ng; n=3 for each group. *P < 0.0001 compared with Rev-erbα - Sp1 -; †P < 0.0001 compared with Rev-erbα - Sp1 + by one-way ANOVA followed by Tukey's *post-hoc* test. (**C**) Validation of the protein expression of mutant Rev-erbα and effects on Cx43 protein expression by immunoblotting. The antibody for Rev-erbα (CST), made against the 20 amino acids between 200 and 300 from the N terminus, including the H region, did not recognize Rev ΔH accordingly. Bmal1 is a positive control of DBD and LBD-indispensable negative regulatory effect by Rev-erbα. (**D**) The A/B region of Rev-erbα transactivated the *Cx43* promoter with Sp1 by itself based on the effect of

HA-Rev AB (a mutant of Rev-erbα with A/B region only). N=3 for each. *P < 0.0001 by one-way ANOVA followed by Tukey's *post-hoc* test. (**E**) The A/B region of Rev-erbα makes a complex with Sp1. DDDDK tagged Sp1 was co-immunoprecipitated using an antibody for HA in HEK293T cells transfected with HA-Rev AB and DDDDK-Sp1. For relative levels, the control without Rev-erbα and Sp1 was set as 1 in **B** and **D**.

Figure 4 The LBD of Rev-erbα competes with the Rev-erbα and Sp1 complex. (**A**) Deletion of the A/B region of Rev-erbα competed with the Rev-erbα and Sp1 complex to transactivate the *Cx43* promoter. N=3 for each. *P < 0.005 compared with Rev-erbα - Sp1 -; †P < 0.005 compared with Rev WT 1.7 Sp1 1.7 by one-way ANOVA followed by Tukey's *post-hoc* test. 1, 37.5 ng; 1.7, 62.5 ng; 2.7, 100 ng; 3.3, 125 ng; 5, 187.5 ng; +, 62.5 ng. (**B**) A mutant of Rev-erbα with only the LBD competes against the WT Rev-erbα as does a mutant with both the DBD and LBDs. N=3 for each. *P < 0.005 compared with Rev-erbα - Sp1 -; †P < 0.005 compared with Rev WT 1.7 Sp1 1.7 by one-way ANOVA followed by Tukey's *post-hoc* test. Error bars represent s.d. in **A** and **B**. The controls without Rev-erbα and Sp1 were set as 1 in **A** and **B**. (**C**) A putative mechanistic scheme for the transactivation of

the *Cx43* promoter by Rev-erbα complexed with Sp1. The A/B region of Rev-erbα interacts with Sp1 as an activation domain and the LBD is a competitive domain. Rev-erbβ, possessing a relatively different A/B region and a similar C terminal LBD, does not activate but competes for transactivation of the *Cx43* promoter.

Figure 1 O. Ogawa *et al*

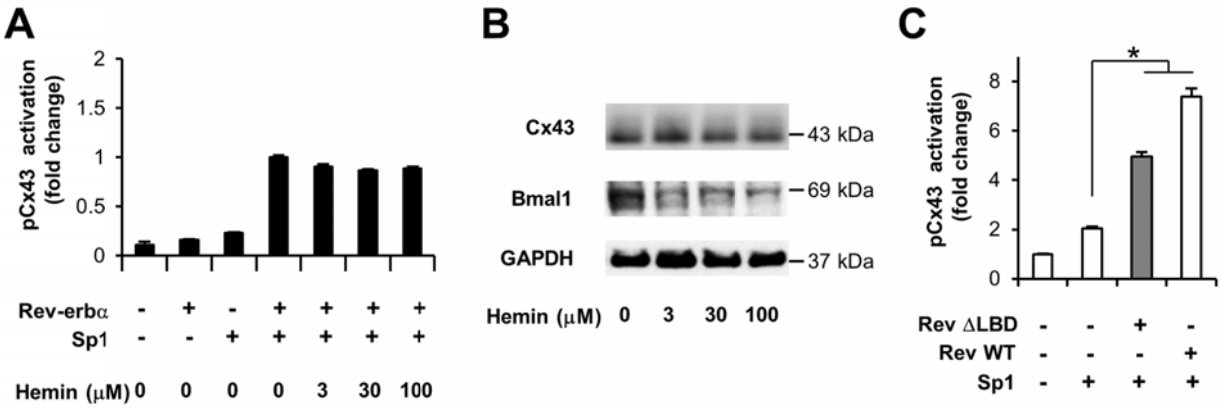


Figure 2 O. Ogawa *et al*

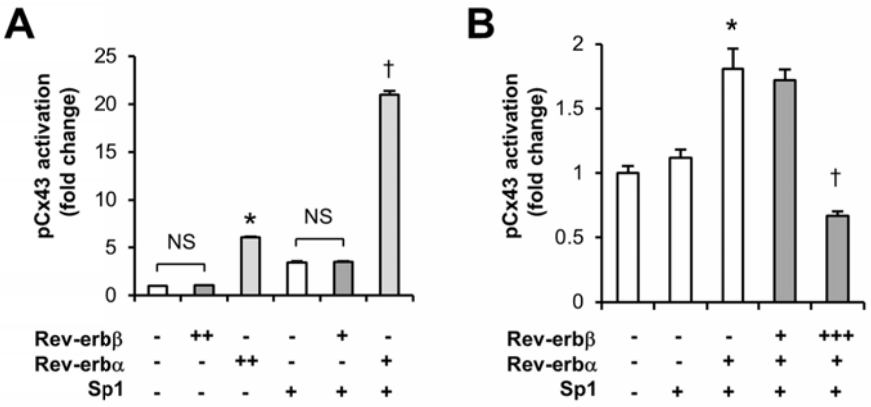


Figure 3 O. Ogawa *et al*

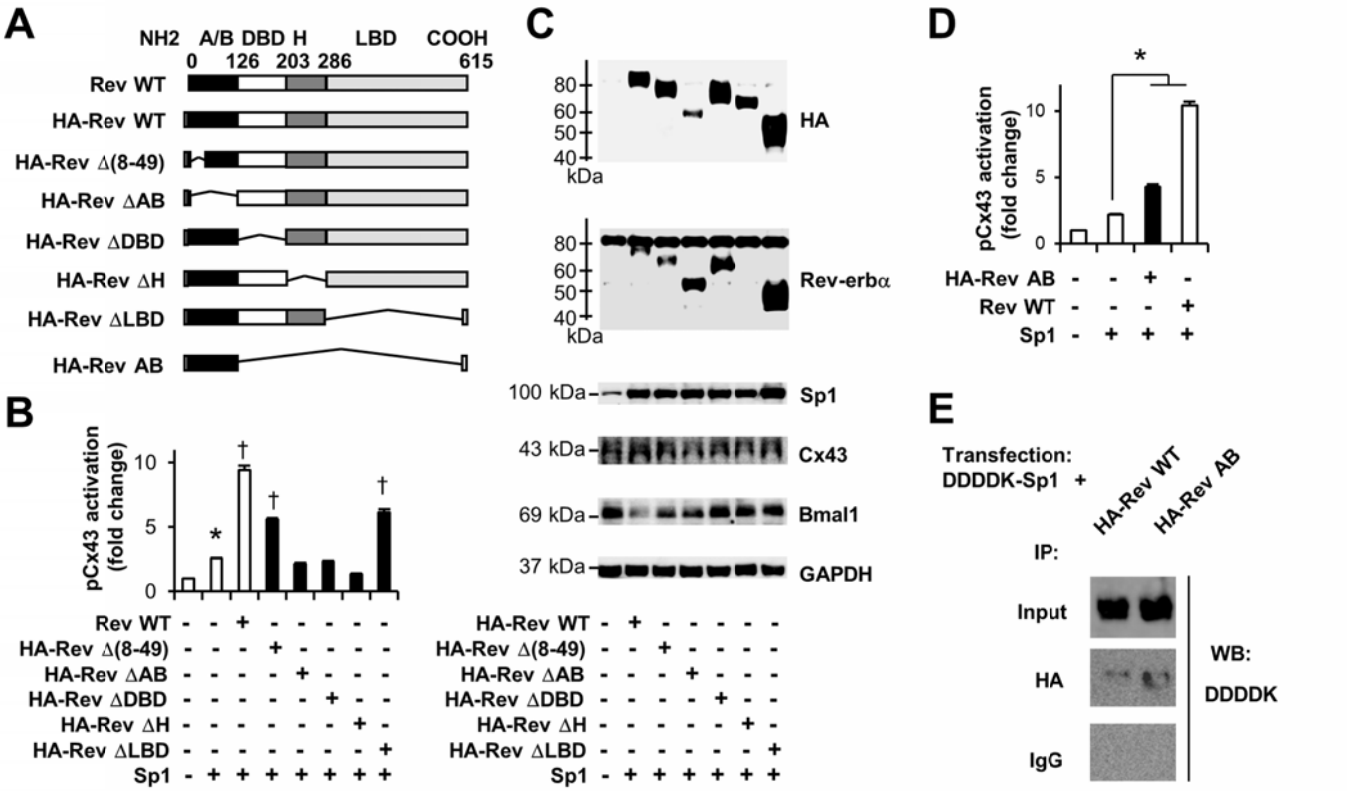
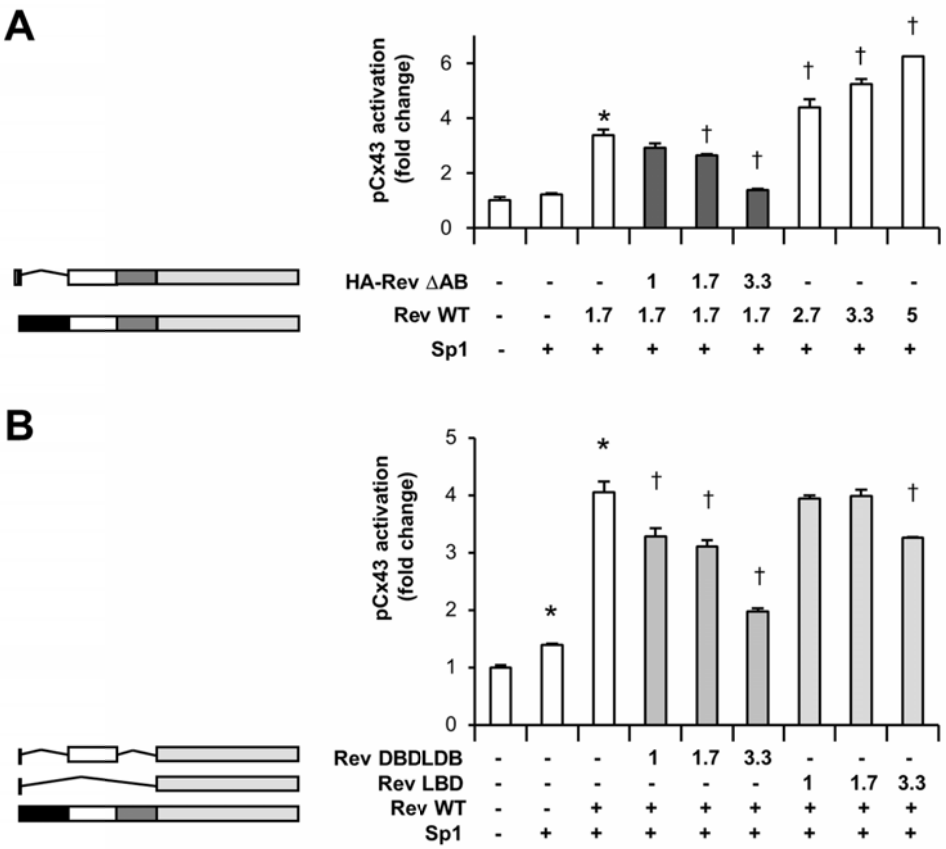


Figure 4 O. Ogawa *et al*



Supplementary Information for

Role of Rev-erbα domains for transactivation of the connexin43 promoter with Sp1

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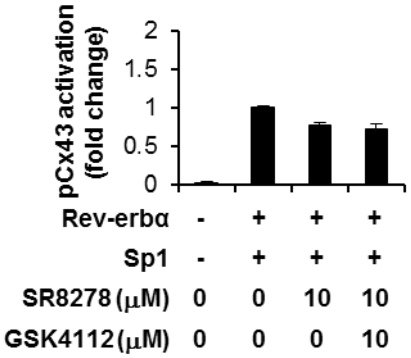
Supplementary Information includes:

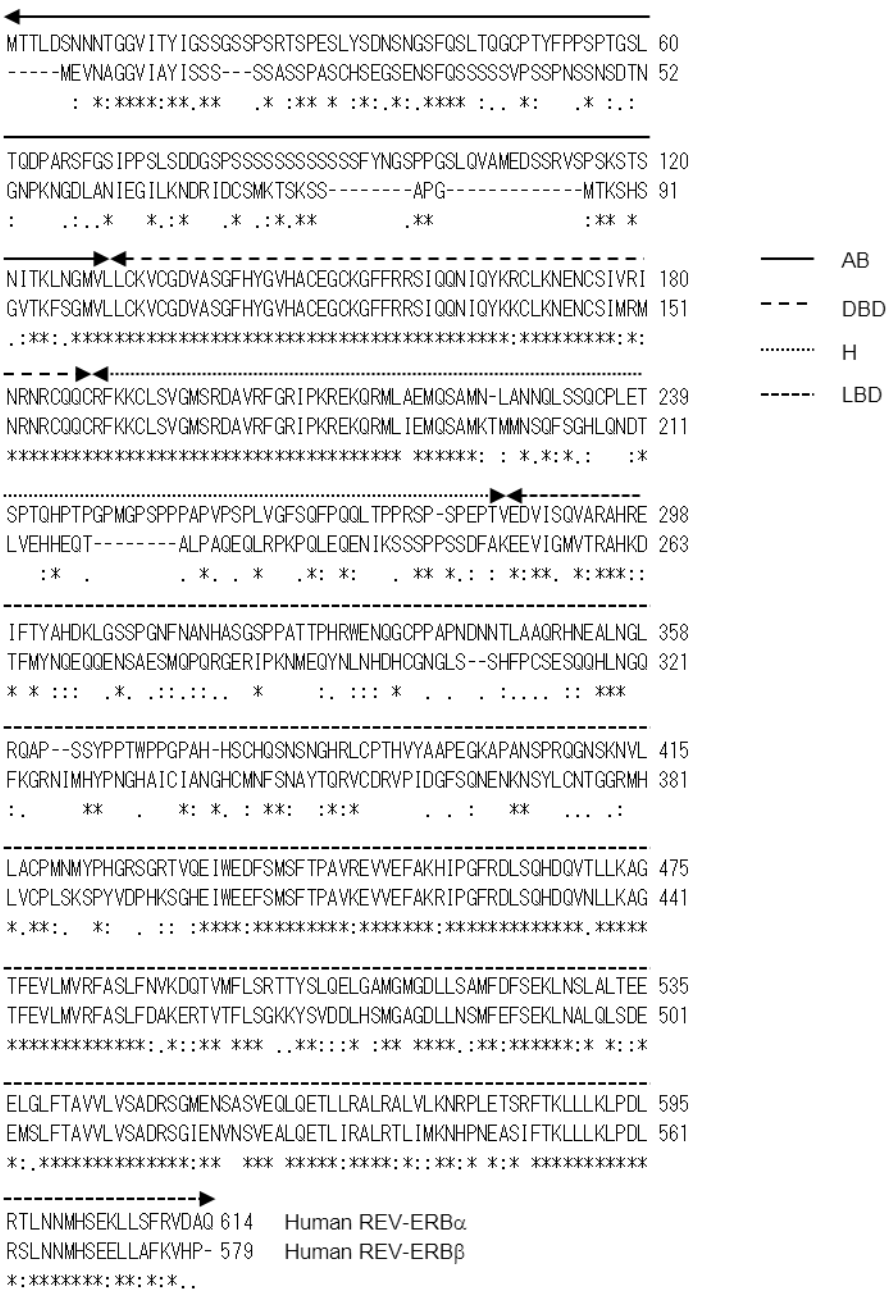
Supplementary Figure S1-S4

Supplementary Figure S1



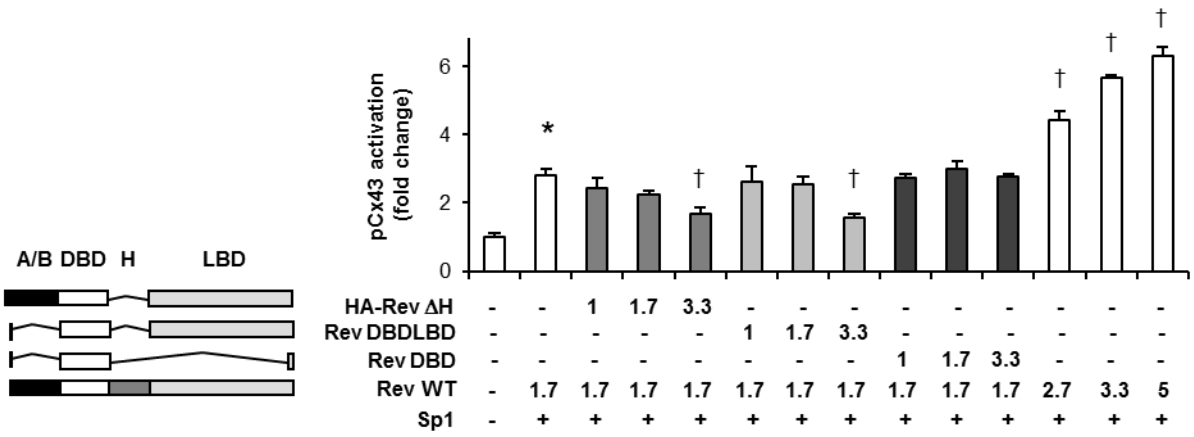
Supplementary Figure S1 | Minor effect of a synthetic antagonist or agonist of REV-ERB on pCx43 activation by the Rev-erbα and Sp1 complex. Addition of SR8278, a synthetic antagonist of REV-ERB, with and without a synthetic agonist GSK4112, did not have major impact on the effect of Rev-erbα and Sp1 on *Cx43* transcription. N=3 for each group. +, 125 ng. Error bars represent s.d. For relative levels, Rev-erbα + Sp1 + without SR8278 and GSK4112 was set to 1.

Supplementary Figure S2



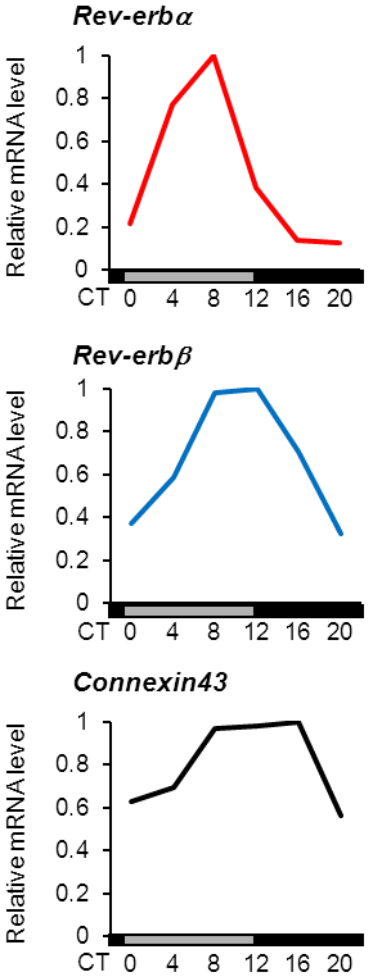
Supplementary Figure S2 | Alignments between human REV-ERBα and REV-ERBβ. Human REV-ERBα and REV-ERBβ have a relatively different A/B region and a similar C terminal LBD analysed by CLUSTAL W. An asterisk, double dots and a dot indicate an identical or conserved residue, a conserved substitution and a semiconserved substitution. AB, A/B region; DBD, DNA binding domain; H, Hinge region; LBD; Ligand biding domain.

Supplementary Figure S3



Supplementary Figure S3 | Mutants of Rev-erbα with the LBD compete against WT Rev-erbα. HA-Rev DBD LBD, a mutant of Rev-erbα only with the DBD and LBD; HA-Rev DBD, a mutant of Rev-erbα only with the DBD. N=3 for each. *P < 0.005 compared with Rev-erbα - Sp1 -; †P < 0.005 compared with Rev WT 1.7 Sp1 1.7 by one-way ANOVA followed by Tukey's *post-hoc* test. 1, 37.5 ng; 1.7, 62.5 ng; 2.7, 100 ng; 3.3, 125 ng; 5, 187.5 ng; +, 62.5 ng. Error bars represent s.d.

Supplementary Figure S4



Supplementary Figure S4 | Temporal gene expressions of Rev-erbα, Rev-erbβ and Connexin43 in the urinary bladder in mice. The max correlation efficiency to 60 phase different cosine curves of Rev-erbα, Rev-erbβ and Conneixn43 are 0.99, 0.98 and 0.86, respectively and the peak phase are CT 7.2, 10.8 and 11.6 respectively. These figures are based on the microarray data deposited in the Gene Expression Omnibus under accession code GSE35795 (ref.17).